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Antibody Protection against Botulinum Neurotoxin Intoxication in Mice[▽]

Luisa W. Cheng,[1]* Larry H. Stanker,[1] Thomas D. Henderson II,[1] Jianlong Lou,[2] and James D. Marks[2]

Foodborne Contaminants Research Unit, Western Regional Research Center, USDA Agricultural Research Service, 800 Buchanan Street, Albany, California 94710,[1] and Department of Anesthesia and Perioperative Care, University of California, San Francisco, San Francisco General Hospital, Room 3C-38, 1001 Potrero Avenue, San Francisco, California 94110[2]

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Adulteration of food or feed with any of the seven serotypes of botulinum neurotoxin (BoNT) is a potential bioterrorism concern. Currently, there is strong interest in the development of detection reagents, vaccines, therapeutics, and other countermeasures. A sensitive immunoassay for detecting BoNT serotype A (BoNT/A), based on monoclonal antibodies (MAbs) F1-2 and F1-40, has been developed and used in complex matrices. The epitope for F1-2 has been mapped to the heavy chain of BoNT/A, and the epitope of F1-40 has been mapped to the light chain. The ability of these MAbs to provide therapeutic protection against BoNT/A intoxication in mouse intravenous and oral intoxication models was tested. High dosages of individual MAbs protected mice well both pre- and postexposure to BoNT/A holotoxin. A combination therapy consisting of antibodies against both the light and heavy chains of the toxin, however, significantly increased protection, even at a lower MAb dosage. An in vitro peptide assay for measuring toxin activity showed that pretreatment of toxin with these MAbs did not block catalytic activity but instead blocked toxin entry into primary and cultured neuronal cells. The timing of antibody rescue in the mouse intoxication models revealed windows of opportunity for antibody therapeutic treatment that correlated well with the biologic half-life of the toxin in the serum. Knowledge of BoNT intoxication and antibody clearance in these mouse models and understanding of the pharmacokinetics of BoNT are invaluable for future development of antibodies and therapeutics against intoxication by BoNT.

Botulinum neurotoxins (BoNTs) are considered some of the most potent toxins known and are potential bioterrorist threat agents. Yet, BoNT serotype A (BoNT/A) and BoNT/B are also used therapeutically in a wide array of medical conditions, such as dystonia and eye disorders like strabismus and blepharospasms, for pain management, and more (3, 25). Thus, there is a need to protect humans and animals against toxin exposure from contaminated food or feed and yet preserve the medical benefits of BoNT. A better understanding of the biology of the toxin, such as toxin distribution and mechanisms of toxin neutralization following intoxication, is needed to aid further development of improved therapies, as well as bona fide use of toxin to treat serious medical conditions.

BoNTs are 150-kDa endopeptidase toxins that are produced by *Clostridium botulinum*, *C. butyricum*, and *C. baratii* (20, 26, 27). The toxin polypeptide is cleaved upon secretion from the cell by bacterial proteases or proteases in the animal host into a disulfide bond-linked dipeptide consisting of a 100-kDa heavy chain (Hc) and a 50-kDa light chain (Lc). The 50-kDa Lc contains the active site or catalytic domain that targets the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor. Specifically, the synaptosome-associated 25-kDa protein (SNAP25) is the target for BoNT/A. Cleavage of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor results in the inhibition of neurotransmitter vesicle docking and subsequent vesicle fusion and release of the neurotransmitter acetylcholine. The toxin Hc contains the cell receptor binding and translocation domains necessary for the uptake of the 150-kDa holotoxin by neurons (18, 24).

Currently, the therapies available for botulism patients are supportive intensive care, such as mechanical ventilation, and antibody treatment postintoxication with equine antitoxins or a botulinum immunoglobulin (BabyBIG) human antibody (3, 6, 9). Drawbacks of those therapies are serious side effects such as serum sickness and anaphylaxis for the use of equine antitoxins and the limited availability of the human antibody product. Neither treatment reverses paralysis completely or immediately, since they act by neutralizing toxin in the circulation. Thus, both must be administered relatively early before toxin uptake by neurons in order to be effective. Currently, there is intense effort to develop better vaccines and antitoxins against BoNTs. Antibodies and other molecular targets, such as small peptides and receptor mimics, and various functional domains of the toxin, such as the catalytic domain and the receptor binding or translocation domain, have previously been developed as therapeutic targets (7, 8, 15, 25, 26).

Studies identifying the antibody binding sites and their toxin neutralization potential have helped identify targets for the design of antibody therapeutics (11). However, to facilitate vaccine approval and development of additional therapeutics, animal model data are needed. Information gleaned from animal models will lead to a better understanding of the efficacy of treatment, as well as define the window of opportunity for

* Corresponding author. Mailing address: Foodborne Contaminants Research Unit, Western Regional Research Center, USDA Agricultural Research Service, 800 Buchanan Street, Albany, CA 94710. Phone: (510) 559-6337. Fax: (510) 559-5880. E-mail: luisa.cheng@ars.usda.gov.

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optimal treatment. Such knowledge will result in the design of better therapeutics and treatment regiments. Thus far, the mouse model of botulism has been used extensively for toxin detection, for toxin concentration standardization, and in the development of therapeutics (9, 12).

We previously described the development of high-affinity monoclonal antibodies (MAbs) F1-2 (against the Hc) and F1-40 (against the Lc) (28). These MAbs were used successfully in detection assays for BoNT/A in complex food matrices. The binding site for MAb F1-40 has been mapped to the exposed loop between the β4 and β5 regions of the Lc. While this site is located on the Lc, it is distal from the catalytic domain. The binding site for MAb F1-2 is conformational and has been mapped to the region between R564 and K595 within the transmembrane domain of the toxin (22, 23).

By using animal studies, one can measure the pharmacokinetics of toxins, the physiological distribution and stability of toxins, and how they are cleared from the system by MAbs. By using ^{125}I-labeled BoNT/A and BoNT/B, the biological half-lives ($t_{1/2}$s) of such toxins have been determined in blood (2, 21). Furthermore, BoNTs were shown not to go through biotransformation during epithelial membrane translocation or by interaction with blood components. Following antibody treatment, toxin was found sequestered mainly in the liver of the animal; however, the mechanism of antibody clearance has not yet been determined.

Here we report the in vivo neutralization of BoNT/A by MAbs in systemic and oral models of botulism. The effects of antibody dosage and timing of administration were tested, and a window of opportunity for MAb rescue following intoxication was defined. To better understand MAb neutralization, the toxin levels in mouse serum over time were determined and compared with the timing of MAb rescue postintoxication. A direct correlation of toxin pharmacokinetics with antibody rescue was found. The individual MAbs also were tested in vitro for enzymatic activity inhibition and blockade of SNAP25 cleavage. These latter studies were designed to mimic conditions found in human intoxication. Increased knowledge of the pharmacokinetics of the toxin and how antibodies clear the toxin will aid in the design of more effective treatments for botulism.

MATERIALS AND METHODS

Materials. Purified holotoxin and complex BoNT/A were purchased from Metabiologics Inc. (Madison, WI) and stored at 4°C and −20°C, respectively. Toxin samples were diluted in phosphate-gelatin buffer (0.028 M sodium phosphate [pH 6.2], 0.2% gelatin). Catalytically inactive BoNT/A holotoxin (ciBoNT/A) was kindly provided by the Smith laboratory (30) and stored at −20°C. Rabbit anti-BoNT/A antibody was purchased from Metabiologics and stored at 4°C. SNAPtide substrate and BoNT/A Lc were purchased from List Biological Laboratories (Campbell, CA) and diluted as suggested by the manufacturer. The MAbs used were F1-2 and F1-40 (28). Antibodies were purified from mouse ascites fluid by protein G affinity chromatography and quantified by using a mini bicinchoninic acid protein assay (Pierce, Rockford, IL). Purified antibodies were diluted in phosphate-buffered saline (PBS; pH 7.4) and stored at −80°C. Female Swiss Webster (18 to 21 g and pregnant) C57BL/6 (embryonic day 18) mice were purchased from Charles River Laboratories (Portage, MI). *Escherichia coli* expressing recombinant human SNAP25bHA was kindly provided by the Barbieri laboratory (5). The Neuro-2A mouse neuroblastoma cell line was purchased from ATCC. Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA).

In vivo neutralization of toxins. For the mouse systemic model, random groups of either 5 or 10 mice were injected intravenously (i.v.) by the lateral tail vein with 100 μl of BoNT/A holotoxin in phosphate gelatin buffer at 1,000 pg/mouse. In the oral model, 10 mice were treated with 6 μg BoNT/A complex in 100 μl phosphate gelatin buffer via gavage with round-end Popper needles. One hundred microliters of the MAbs at the indicated concentrations was diluted in PBS and introduced i.v. into mice at the indicated times either pre- or postintoxication. Mice were monitored for at least 7 days for intoxication symptoms or death. The weight and health status of mice were observed over a 21-day period. All of the in vivo protocols described here were approved by the Animal Care and Use Committee of the USDA, Western Regional Research Center, Albany, CA.

In vitro assays for toxin activity. Toxin cleavage of SNAPtide (fluorescein isothiocyanate–4,4-dimethylamino-azobenzene-4′-carboxylic acid) by BoNT/A holotoxin or Lc was performed as suggested by the manufacturer (List Biological Laboratories, Inc.), with minor modifications. Briefly, 5, 10, or 20 nM holotoxin or Lc was pretreated with 2 μg MAbs or a rabbit anti-BoNT/A antibody for 15 min at 37°C (control samples were not pretreated with MAbs), followed by the addition of 8 μM SNAPtide substrate in a 100-μl reaction mixture (20 mM HEPES [pH 7.4], 1.25 mM dithiothreitol, 0.3 mM $ZnCl_2$, 0.2% Tween 20). Triplicate reactions were run in 96-well black plates (Costar) with clear flat bottoms. Reactions were carried out for 30 min to 5 h at 37°C. Fluorescence was measured with a Perkin-Elmer Victor-2 microplate reader. Graphs shown were derived from 3-h reactions.

Recombinant human SNAP25bHA protein was expressed from the *E. coli* strain as previously described (5), and purified with a glutathione-Sepharose column by using reagents and protocols from GE Healthcare. SNAPtide reaction mixtures consisting of 0.5 to 1 μg of purified GstSNAP25bHA protein in the presence or absence of 10 nM BoNT/A in the presence or absence of 2 μg MAbs in 10 μl of HEPES buffer (described above) were incubated for 15 min at 37°C. The reaction was then stopped by the addition of 4× LDS buffer (Invitrogen) to give a final concentration of 1×, followed by heat inactivation (90°C for 10 min). Products were then separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with NuPAGE 10% Bis-Tris gels (Invitrogen), followed by Coomassie blue staining or Western blotting. For Western blot assays, cleaved or uncleaved recombinant GstSNAP25bHA was visualized by staining with a primary goat anti-glutathione *S*-transferase antibody (GE Healthcare) diluted 1:5,000 in 5% milk–Tris-buffered saline–0.05% Tween 20 buffer, followed by a horseradish peroxidase (Pierce)-conjugated rabbit anti-goat immunoglobulin G secondary antibody. Stained gels and Western blot assays were analyzed with a FlurChem SP AlphaImager (Alpha Innotech, San Leandro, CA). Molecular weight standards were purchased from Invitrogen. Statistical significance of data collected was determined with unpaired *t* tests with Prism 4 statistical software (GraphPad Software Inc., San Diego, CA).

Serum toxin measurements. The concentrations of BoNT/A and ciBoNT/A in mouse serum were measured with a SECTOR Imager 2400 from Meso Scale Diagnostic (MSD). Blood was collected from random groups of five mice (body weights of 19.5 to 20 g) via cardiac puncture into serum collection tubes with gel separators (BD) at each time or dosage point. Blood was incubated on ice for at least 30 min before separation by centrifugation at 3,000 × *g* for 10 min. Collected serum were aliquoted and stored at −80°C before analysis. MSD assays used human MAb ING2 for toxin capture and SULFO-TAG-labeled MAb CR2 as a reporter (13, 16). Assay protocols were optimized after screening and comparing eight different MAbs specific for BoNT/A. Briefly, ING2 diluted in PBS at a concentration of 0.5 μg/ml was used to coat an MA2400 96 standard plate at 30 μl/well overnight at 4°C. After blocking with 2% milk powder in PBS for 30 min, 1:1-diluted serum samples or toxin standards were added to each well. Every sample or toxin standard was measured in triplicate by using three wells on the same plate. The toxin standard was purified BoNT/A (Metabiologics) or purified ciBoNT/A serially diluted in normal mouse serum from a starting concentration of 1.6 μg/ml. The plate was then shaken vigorously at room temperature for 1 h and washed three times with PBS-Tween 20, followed by three washes with PBS, before the addition of SULFO-TAG-labeled CR2 and incubation for 1 h. Plates were washed again as described above, the MSD reading buffer was then added, and plates were read in a SECTOR Imager 2400 instrument. The final result was processed with the MSD DISCOVERY WORKBENCH software DATA ANALYSIS TOOLBOX. The concentration of ciBoNT/A in serum samples was determined from standard curves (see Fig. 4A) made with known ciBoNT/A standards diluted in normal mouse serum.

Primary neuronal cell culture assay for toxin. Cortical neurons were isolated from embryonic day 18 embryos from C57BL/6 mice. Embryos were dissected, and whole brains were isolated, separated into two hemispheres, and placed onto dishes containing cold Hanks balanced salt solution. Cortex tissues were separated from internal tissues and meninges and transferred to

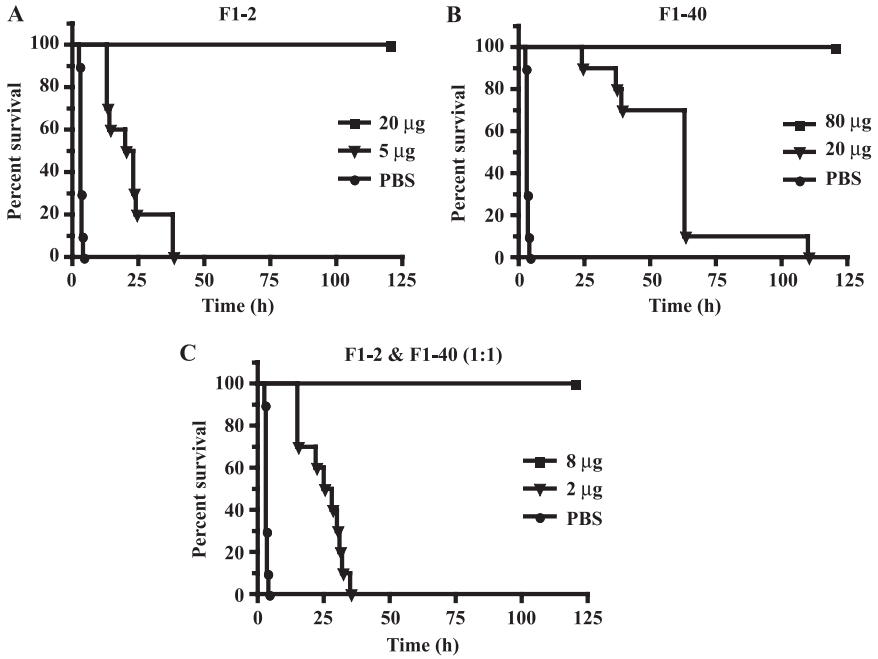


FIG. 1. MAb neutralization of BoNT/A in a systemic mouse model. Diagrams show survival curves of mice treated with BoNT/A holotoxin at 1,000 pg/mouse (143 mouse i.v. LD_{50}). The percent survival of mice treated with different doses of F1-2, a MAb against the Hc of BoNT/A (A); F1-40, a MAb against the Lc of BoNT/A (B); or HL MAbs, a 1:1 combination of these two MAbs (C), at 1 h prechallenge with BoNT/A was plotted over time. Control mice were treated with PBS i.v. instead of MAbs before intoxication.

sterile 50-ml tubes containing fresh Hanks balanced salt solution. Cortical tissues were then triturated with a flame-polished glass pipette. Large precipitates were allowed to settle, and the cell suspension was removed and centrifuged at 1,000 × *g* for 5 min; the supernatant was discarded, and the cell pellet was resuspended in culture medium (neurobasal medium supplemented with B27, GlutaMAX, and penicillin-streptomycin). All culture media and supplements were purchased from Invitrogen, except where indicated otherwise. Cells were then diluted and plated onto poly-L-lysine (Sigma)-coated 12-well dishes at 200,000 cells per well. Cells were incubated at 37°C in a moist 5% CO_2 atmosphere. Fourteen- to 16-day-old cultured neurons were left untreated or treated with 1,000 pg/ml BoNT/A in the presence or absence of 20 μg of individual F1-2, F1-40, or a 1:1 combination of both MAbs (HL MAbs) for 8 h. Cells were washed in 2× PBS before lysis with 1 ml lysis buffer (PBS with 1% Triton X-100 and 0.05% SDS), followed by trichloroacetic acid (10%) precipitation. Trichloroacetic acid precipitates were washed with acetone, air dried, and resuspended in 100 μl 1× LDS buffer. Cleavage of SNAP25 was monitored by SDS-PAGE (12% Novex Bis-Tris gels), followed by Western blotting with a primary rabbit anti-SNAP25 antibody (Sigma) and a goat anti-rabbit immunoglobulin G secondary antibody conjugated to horseradish peroxidase (Pierce).

RESULTS

Toxin neutralization in a systemic mouse model. We sought to measure the toxin neutralization characteristics of well-defined individual MAbs and combinations of MAbs against BoNT/A in an in vivo model of botulism. Two MAbs, F1-2 (specific against the Hc of BoNT/A) and F1-40 (specific against the Lc of the toxin), were tested for therapeutic protection against lethal doses of BoNT/A holotoxin in a systemic mouse model of intoxication. The MAb dose response and the effects of different administration times were determined. Following the delivery of a lethal dose of BoNT/A (1,000 pg/mouse or about 143 mouse i.v. 50% lethal doses [LD_{50}]), MAbs were delivered i.v. and the animals were monitored over time. In the absence of MAbs, intoxicated mice treated with PBS alone died within 2.5 to 3.5 h (Fig. 1). Mice pretreated with 20 μg of F1-2 or 80 μg of F1-40 at 1 h preintoxication were completely protected from death (Fig. 1A and B). Doses of 5 and 20 μg of each respective MAbs were partially protective, as evidenced by an increased time to death. Use of a combination (1:1) of F1-2 and F1-40 (referred to from here on as HL MAbs) enhanced survival. Pretreatment with a total of 8 μg of HL MAbs prevented death in mice, while as little as 2 μg of HL MAbs increased the time to death (Fig. 1C).

In addition to the MAb dosage, the effect of the timing of MAb administration on animal survival was also evaluated. However, percentages of animal survival do not tell the complete story. As is often the case, BoNT/A-treated mice might show signs of illness (either physiological or weight loss) but recover after a few days. Thus, mice were also monitored for signs of botulism and weight loss for 3 to 4 weeks after toxin treatment (Fig. 2).

Mice treated with 10 μg of HL MAbs at 5 min before a BoNT/A challenge survived the challenge but showed slight signs of illness and weight loss in the first 2 to 3 days compared to buffer-treated control animals (Fig. 2A versus B). In contrast, mice treated with the same dosage of BoNT/A but at 5 min postintoxication exhibited signs of botulism, as denoted by weight loss and death (Fig. 2C and 3A). When the HL MAb dosage was increased to 80 μg and administered 5 min postintoxication, all of the mice were protected from death and showed minimal physiological symptoms or weight loss (Fig. 2D). All of the mice that survive a toxin challenge will eventually recover their weight and health to look indistinguishable from control mice not treated with toxin.

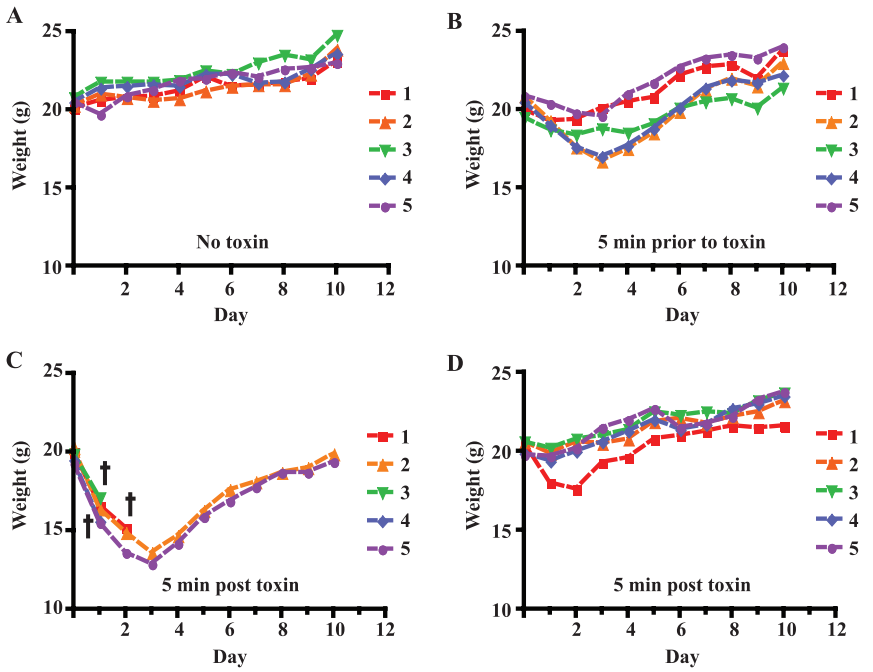


FIG. 2. Effects of MAb injection time and dosage on toxin neutralization. Weights of five individual mice treated i.v. with 1,000 pg/mouse BoNT/A were plotted for 10 days. Mice were treated with PBS (A), BoNT/A with 10 μg of HL MAbs 5 min preintoxication (B), BoNT/A with 10 μg of HL MAbs 5 min postintoxication (C), or BoNT/A with 80 μg of HL MAbs at 5 min postintoxication (D). Crosses represent mice that did not survive the toxin challenge.

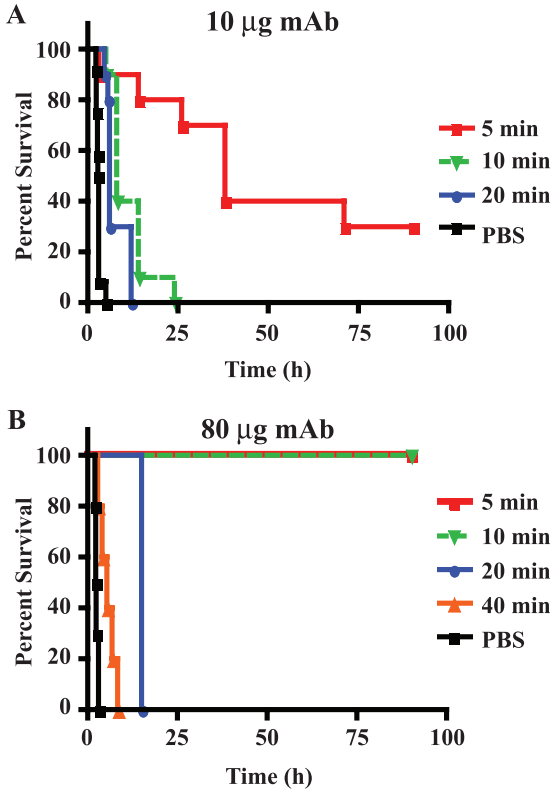


FIG. 3. Window of opportunity for rescue from systemic intoxication with BoNT/A. The percent survival of groups of 10 mice challenged i.v. with 1,000 pg of BoNT/A holotoxin, followed by PBS or MAb treatment with (A) 10 μg of HL MAbs or (B) 80 μg of a MAb combination administered i.v. at the indicated times was plotted over time.

A window of opportunity for the rescue of mice from systemic intoxication was observed when treating mice with low (10 μg) and high (80 μg) doses of HL MAbs at increasingly longer postintoxication times (Fig. 3A and B). With a low-dose MAb treatment postintoxication, most mice were not protected from death. However, a slight increase in the time to death was observed in animals treated with HL MAbs at up to about 20 min postintoxication (Fig. 3A). Treatment with a high dose of HL MAbs completely rescued mice from death if the antibodies were administered at up to 10 min postintoxication, and an increase in the time to death was observed when the antibodies were administered at up to about 20 min postintoxication (Fig. 3B). Mice treated at 40 min or later postintoxication (with either high- or low-dose HL MAbs) displayed no significant increase in the time to death compared with that of PBS-treated mice (Fig. 3A and B).

Postintoxication serum toxin concentration. A clearer understanding of toxin pharmacokinetics in serum is needed in order to gain a better understanding of antibody neutralization and how the windows of opportunity for rescue correlate with levels of toxin in the animal bloodstream. Meaningful in vivo pharmacokinetic data have been difficult to obtain from mice because only picogram amounts of BoNT/A are necessary to kill a mouse and there is a lack of sensitive methods for the detection of such minute quantities of toxin, other than radiolabeling, especially in complex matrices such as serum. A new method for BoNT/A detection in animal serum by an electrochemiluminescence enzyme-linked immunosorbent assay method with a commercially available SECTOR Imager 2400 instrument from MSD was thus developed. With a pair of extremely high-affinity human MAbs against BoNT/A

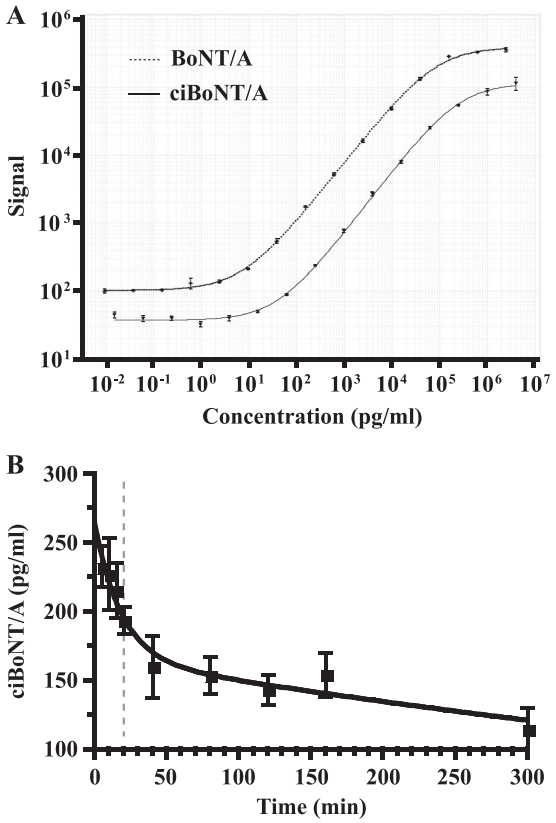


FIG. 4. Systemic pharmacokinetics of ciBoNT/A. (A) A new method for BoNT/A detection in mouse serum with a commercially available SECTOR Imager 2400 instrument from MSD was developed. With human anti-BoNT/A MAbs, ING2 for capture, and SULFO-TAG-labeled CR2 for detection, the concentration of unknown ciBoNT/A was determined from standard curves made with known ciBoNT/A standards serially diluted in normal mouse serum. (B) Random groups of five mice were injected with 1,000 pg/mouse of ciBoNT/A. Serum ciBoNT/A content was determined over time (5, 10, 20, 30, 40, 80, 120, 160, and 300 min postintoxication). Gray bars denote the window of opportunity for rescue or for a delay of botulism lethality. Each data point in the graph represents a mean ± the standard error of the mean.

with nonoverlapping epitopes for capture (ING2) and detection (SULFO-TAG-labeled CR2) (13, 16), as little as 10 pg/ml of BoNT/A can be detected and the range of detection in mouse serum is 10 pg/ml to 100 ng/ml (Fig. 4A).

In addition to increased assay sensitivity, the survival time of mice needs to be prolonged in order to better study toxin pharmacokinetics. A typical intoxication with 1,000 pg BoNT/A holotoxin per mouse will cause death in about 2.5 to 3.5 h. In order to obtain meaningful and long-range toxin biological $t_{1/2}$s, a recombinant nontoxic BoNT/A holotoxin (ciBoNT/A) surrogate was used instead of the BoNT/A holotoxin for the determination of pharmacokinetics. ciBoNT/A contains point mutations (amino acid substitutions H223A, E224A, and H227A) that abrogated the catalytic activity of the endopeptidase (30). In contrast to BoNT/A holotoxin-treated mice, mice administered 1,000 pg of ciBoNT/A did not show any signs of toxicity (data not shown). In mouse serum, MAbs ING2 and CR2 recognized ciBoNT/A with binding kinetics and equilibrium dissociation constants similar to those with which they bind the BoNT/A holotoxin standard (data not shown), resulting in a standard curve similar to that for BoNT/A (Fig. 4A).

Systemic injections of mice with ciBoNT/A under conditions identical to those used in the systemic models evaluating MAb neutralization of BoNT/A holotoxin were performed. Random sets of five mice were challenged i.v. with a 1,000-pg/mouse dose of ciBoNT/A. Serum was collected from each set of mice over time, and the levels of ciBoNT/A were determined with the MSD instrument. Figure 4B shows a diagram of serum concentrations of ciBoNT/A plotted over time. We observed a rapid drop in blood toxin levels within the first 20 min of toxin introduction, followed by a slower rate of toxin decline in the blood. This initial phase of rapid toxin decline represents the redistribution or alpha $t_{1/2}$ ($t_{1/2\alpha}$). The $t_{1/2\alpha}$ of ciBoNT/A was determined to be 63 min and most probably represents the period when the toxin is redistributed to tissues and extracellular spaces and is taken up by neurons. A slower rate of toxin decline follows the $t_{1/2\alpha}$; this phase or beta $t_{1/2}$ ($t_{1/2\beta}$) was determined to be about 450 min (Fig. 4B). The $t_{1/2\beta}$ likely represents clearance of the toxin from the blood. Both $t_{1/2\alpha}$ and $t_{1/2\beta}$ were comparable to the 61-min $t_{1/2\alpha}$ and 600-min $t_{1/2\beta}$ of BoNT/B and the overall BoNT/A $t_{1/2}$ of 231 min previously determined in mice with radiolabeled toxins (2, 21).

Toxin neutralization in an oral mouse model. In 2007, 18% of the known natural noninfant botulism cases in the United States occurred via the oral route, usually by consumption of contaminated foods (National Botulism Surveillance, Centers for Disease Control and Prevention; http://www.cdc.gov/nationalsurveillance/botulism_surveillance.html). However, BoNTs also pose bioterrorism concerns and scenarios of intentional contamination of the food supply have been advanced (31). Increased knowledge of the oral intoxication process and the biology of BoNTs in an animal following oral intoxication is therefore needed. We developed an oral mouse model to simulate food-borne intoxication in order to study the ability of MAbs to neutralize the toxin. We previously demonstrated (10) that 17 times more BoNT/A holotoxin than BoNT/A complex was needed to cause death when intoxication occurred via the oral route, presumably due to the protection from acid and peptic degradation by the neurotoxin-associated proteins. Since BoNT is secreted by *Clostridium* as a complex, we used the toxin complex in all experiments with the oral model. Results from these experiments are summarized in Fig. 5. The BoNT/A complex was delivered directly into the stomachs of mice via gavage. Mice received 6 μg or about 3.5 oral LD_{50} of the BoNT/A complex, followed by i.v. injection of 20 μg of HL MAbs at the postexposure times indicated (Fig. 5A). Injection of 20 μg of HL MAbs i.v. within 4 h conferred complete protection (defined as no deaths among the treated mice). Mice treated within 4 h postintoxication were mostly healthy, showing no signs of botulism, and gained weight similarly to non-toxin-treated mice (Fig. 5B to F). Treatment with the same doses of HL MAbs at 6 and 8 h postintoxication reduced mortality to 40% and 60%, respectively (Fig. 5A). Surviving mice at these latter time points showed increasing signs of illness, as illustrated by the larger number of mice that lost weight or died. Mice that survive will recover completely over time (Fig. 5D and E). In contrast, death occurred in about 2 days in control mice receiving an injection of PBS rather than MAbs (Fig. 5A).

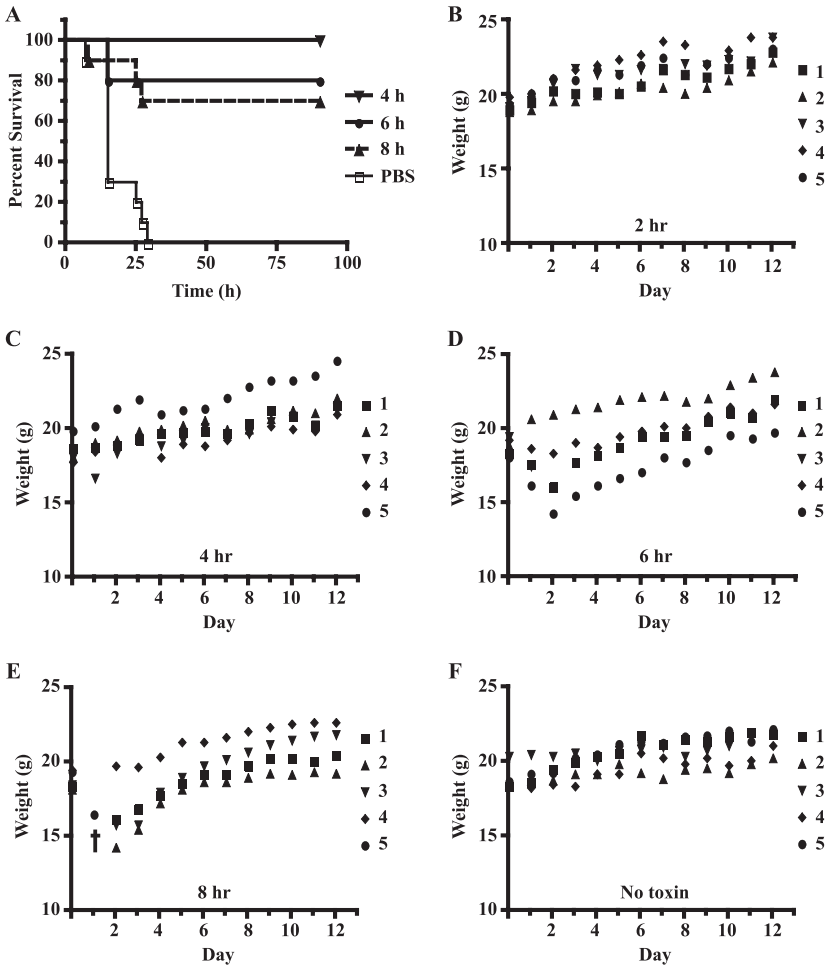
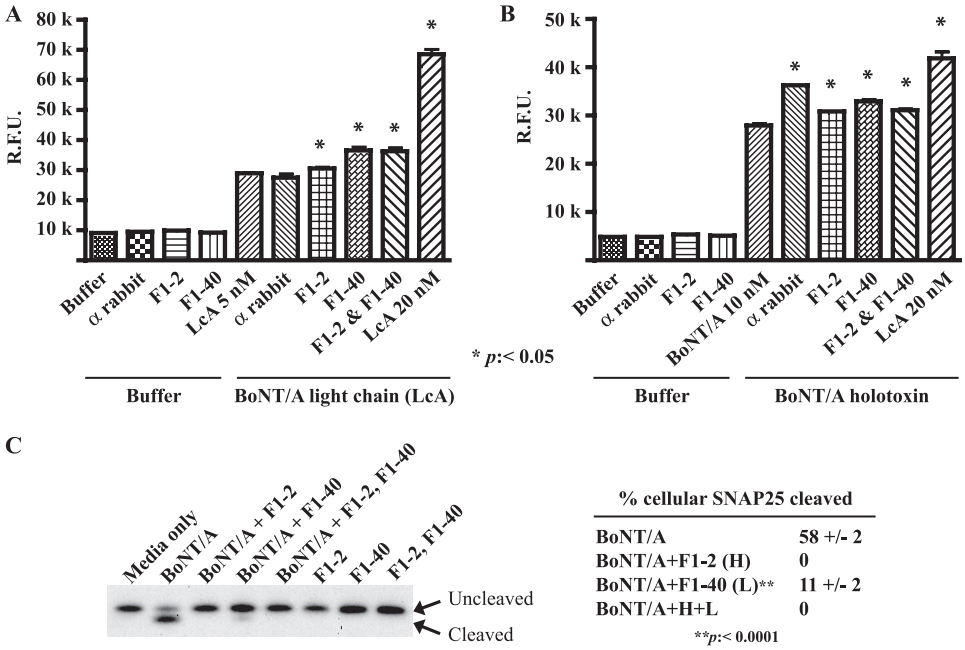


FIG. 5. MAb neutralization of BoNT/A holotoxin in an oral mouse model. Mice were administered 6 μg/mouse or about 3.5 mouse oral LD_{50} of BoNT/A complex by gavage, followed by a rescue with 20 μg of HL MAbs by i.v. administration at the indicated times. (A) The percent survival of mice was plotted over time. Control mice (no toxin) were treated with equal volumes of phosphate gelatin buffer instead of toxin. (B to F) Weights of toxin-challenged mice treated with HL MAbs and of nontreated mice were plotted over time. Crosses represent mice that did not survive the toxin challenge.

The F1-2 and F1-40 MAbs do not inhibit BoNT/A catalytic activity but block intracellular SNAP25 cleavage. MAbs can neutralize BoNTs by at least several possible mechanisms: (i) blocking the catalytic activity of the toxin, (ii) blocking toxin binding to neurons, or (iii) enhancing clearance by opsonization, etc. To determine whether MAb F1-2 or F1-40 inhibited catalytic activity, SNAPtide, a commercially available fluorescent peptide containing the BoNT/A cleavage site of SNAP25, was used in in vitro assays to measure endopeptidase activity. Addition of the individual MAbs, the HL MAbs, or a polyclonal rabbit anti-BoNT/A antibody did not inhibit the endopeptidase activity of either the BoNT/A Lc or the holotoxin (Fig. 6A and B). SNAPtide incubated with antibodies but without the holotoxin or Lc showed no cleavage activity. The presence of MAbs or antibodies to BoNT/A enhanced substrate cleavage slightly. This slight substrate cleavage enhancement was also observed in a different in vitro assay with the recombinant human GstSNAP25bHA substrate. BoNT cleaved the C terminus of recombinant GstSNAP2b5HA (9 amino acids from the C terminus of SNAP25b and an 11-amino-acid-long hemagglutinin tag at the C terminus of this substrate), yielding a 20-amino-acid-smaller polypeptide that is visible in Coomassie-stained gels or on Western blots probed with an anti-glutathione *S*-transferase antibody (5). Again, the MAbs alone did not cleave the SNAP25 substrate, but combined with the toxin, a slight enhancement of cleavage is observed compared to toxin-only cleavage levels (data not shown), confirming the SNAPtide results.

MAbs were also tested for the ability to inhibit toxin binding and subsequent cleavage of intracellular SNAP25 in primary mouse cortical neurons. Since binding of individual MAbs or HL MAbs did not occlude the active site of the endopeptidase or reduce its enzymatic activity in vitro, any inhibition of SNAP25 cleavage when MAbs were added would be due to a block in toxin cell entry or delivery to where SNAP25 is compartmentalized. Incubation of primary mouse cortical neurons in the presence of BoNT/A with F1-2, F1-40, or the HL MAbs inhibited intracellular SNAP25 cleavage (Fig. 6C). MAb F1-40 (the anti-Lc MAb) was less efficient than F1-2 (the anti-Hc MAb) at inhibition of cleavage. Incubation with individual MAbs or HL MAbs without any toxin yielded no SNAP25 cleavage.



% cellular SNAP25 cleaved	
BoNT/A	58 +/- 2
BoNT/A+F1-2 (H)	0
BoNT/A+F1-40 (L)**	11 +/- 2
BoNT/A+H+L	0

**p:< 0.0001

FIG. 6. MAbs F1-2 and F1-40 do not inhibit BoNT/A endopeptidase activity but prevent intracellular SNAP25 cleavage in primary neurons. Endoprotease activities of (A) the BoNT/A Lc domain and (B) the BoNT/A holotoxin were measured with the in vitro SNAPtide assay. The individual MAbs, a combination of MAbs F1-2 and F1-40, and a commercial polyclonal rabbit anti-BoNT/A antibody were added to the reaction mixtures to test for endopeptidase inhibitory activity. In control reaction mixtures, buffer substituted for toxin. Asterisks denote cleavage levels that were statistically significantly different ($P < 0.05$) from the amount of cleavage in reaction mixtures with toxin lacking antibodies. (C) MAbs F1-2 and F1-40 inhibited intracellular SNAP25 cleavage in primary mouse cortical neurons. Fourteen- to 16-day-old neurons were left untreated or treated with BoNT/A holotoxin in the presence or absence of individual MAbs or a combination of MAbs. Cleavage of intracellular SNAP25 by BoNT/A at the C terminus generated a smaller protein fragment that can be separated by 12% SDS-PAGE and visualized after Western blotting with a rabbit anti-SNAP25 antibody. The percentage of cleaved SNAP25 was determined for reaction mixtures with a visible cleavage fragment (mean ± standard error of the mean, $n = 5$). R.F.U., relative fluorescence units.

DISCUSSION

Development of vaccines and therapies for botulinum intoxication has been an active area of research. Despite the successful clinical use of current equine and human immunoglobulin (BabyBIG) antibody therapies (14, 29), little information is available on the mechanisms of antibody neutralization and the efficacy of MAb toxin neutralization in in vivo systems. In this study, the ability of MAbs to neutralize BoNT/A was modeled in both the systemic and oral mouse models of botulism. We also sought to dissect the contribution of two well-characterized MAbs: F1-40, a MAb against the Lc of BoNT/A that does not bind the catalytic domain, and F1-2, a MAb against the Hc of BoNT/A with a conformational epitope in the translocation domain of the toxin (22, 23). Toxin neutralization studies with mice in which antibody was administered both pre- and postintoxication by i.v. injection revealed that the individual MAbs binding to either the toxin Lc or Hc domain could fully protect mice from death at low toxin challenge doses (Fig. 1A and B). As reported by others, a combination of MAbs against multiple sites of BoNT increased neutralization activity (17). Similarly, combining equal ratios of F1-2 and F1-40 increased the neutralization potential (Fig. 1C), presumably due to MAb binding of the toxin at multiple sites, enabling a synergistic effect of antibody protection either by steric hindrance of receptor binding or by enhancing immune clearance from the blood.

In addition to antibody dosage, the timing of antibody administration also was important. By administering MAb combinations at various times before and after toxin exposure, a window of opportunity for effective MAb toxin neutralization was identified (Fig. 3). Mice treated with a high lethal dose of BoNT/A (143 i.v. LD_{50}) could be rescued completely from death if treated with HL MAbs up to 10 min after i.v. intoxication, and prolonged survival was observed if mice were treated with antibodies up to about 20 min after i.v. intoxication. The data indicate that once large amounts of toxin enter the bloodstream, the window of opportunity to neutralize it is small. However, the size of the survival window can be dependent on the toxin dosage and the neutralization strength of the MAbs used. Lower lethal dosages of toxin and stronger neutralization combinations of MAbs will lead to a slightly larger rescue window (data not shown).

In order to properly understand antibody neutralization, we needed to have a clearer picture of the in vivo biologic $t_{1/2}$ of BoNT/A holotoxin. Toxin quantification in in vivo systems has been hampered by the lack of sensitive methods for detecting toxins in animal serum and organs and the fact that it is not possible to get later time points because the mice die of intoxication. We therefore developed a sensitive electrochemiluminescence-based enzyme-linked immunosorbent assay using the very high-affinity MAbs CR2 and ING2 (13, 16). With this assay, the serum $t_{1/2}$ of ciBoNT/A was determined (Fig. 4) and

found to be comparable to those measured for BoNT/B by radiolabeling and toxin inactivation (2). The serum pharmacokinetic data for ciBoNT/A showed a remarkably strong correlation with the MAb rescue profiles after i.v. intoxication (Fig. 3 and 4B). In our studies, the toxin appeared to be quickly redistributed from the serum following systemic exposure. The initial or $t_{1/2\alpha}$ phase of ciBoNT/A in mice correlated well with the short 10-min time frame for MAb rescue from death and an increase in the time to death after MAb administration at up to 20 min postinjection.

Together, the pharmacokinetic and MAb toxin neutralization data revealed a window of opportunity for rescue after i.v. exposure. The early or $t_{1/2\alpha}$ phase correlates with toxin that is being distributed and sequestered into extracellular spaces or absorbed into neurons. These results together indicate the challenges of using an i.v. model for evaluating antitoxins; the window of opportunity for treatment is too small. The remaining toxin in the serum was cleared slowly, with an elimination $t_{1/2}$ of about 4 h, consistent with findings by other groups using different methods (2). This persistent pool of toxin is the main target of current antitoxin therapies. In food-borne cases of botulism, equine antitoxin therapy is recommended immediately upon the onset of neurological symptoms. Administration of antitoxin therapy improved recovery times and shortened hospital stays even in patients with late administration of antitoxins (3, 29).

The mouse i.v. model successfully mimicked systemic intoxication and provided information on the biologic $t_{1/2}$ of BoNT/A in the bloodstream. However, little is known about the progression and distribution of BoNT/A following oral intoxication and no work had been done to study antibody neutralization following oral uptake of BoNTs. Compared to systemic intoxication, relatively large amounts of toxin must be ingested to cause disease (10, 19). Even though a BoNT/A complex was used for oral intoxication, BoNT holotoxin alone is believed to be the form found in the bloodstream, as the BoNT/A complex is thought to dissociate in blood because of its high hemagglutinin content, and the holotoxin alone is sufficient for translocation and no accessory protein is required (21, 26). Most of the toxin is likely degraded or shed from the system, and little is absorbed. Based on how well the systemic MAb BoNT/A neutralization data mirrored the serum pharmacokinetics of toxin in the i.v. model and assuming that orally ingested BoNT/A would have to reach the bloodstream to cause disease, some limited predictions can be made from the data on antibody neutralization during oral intoxication. Mice orally treated with the BoNT/A complex did not show signs of botulism or weight loss if treated with HL MAbs before 4 h after intoxication (Fig. 5). Thus, the toxin likely does not accumulate in the blood system in adequate amounts to intoxicate until 4 to 6 h after oral intoxication, when some animals later exhibit weight loss (Fig. 5B to F). HL MAb cocktails neutralized orally ingested BoNT/A and both prevented and delayed death if administered at up to about 8 h postintoxication. This is in sharp contrast to the much smaller window of opportunity for protection against systemic intoxication of about 10 min (Fig. 3 and 5A). The long lag time between ingestion of the toxin and when the toxin accumulates to lethal levels in the bloodstream likely corresponds to the time necessary for the toxin to transit from the stomach, traffic through cells of the intestinal tract, be taken up by receptor-mediated endocytosis, be translocated across the intestinal epithelial cells, and subsequently enter the bloodstream and extracellular spaces. Once BoNT reaches the bloodstream, it would likely follow the systemic biological $t_{1/2}$ pattern, where toxin is quickly absorbed (Fig. 4B). However, for both systemic and oral mouse antibody neutralization models, no MAb rescue was observed when MAbs were administered after visible symptoms of botulism (such as slowness, limping, etc.) became apparent.

Attempts have been made by our group to determine serum pharmacokinetics after oral intoxication; however, the distribution of toxin following oral intoxication was difficult to pinpoint for technical reasons. Absorption would be influenced by the digestion or absorption of the toxin, and this may vary with the individual mouse. Increasingly sensitive methods for toxin detection will, in the near future, allow real-time quantitation of toxin levels in a single animal over time. One such sensitive method is the assay with a large immunosorbent surface area (4). This assay involves bead-based capture of the toxin, followed by measurement of the toxin's intrinsic metalloprotease activity with a fluorogenic peptide substrate, and has a low limit of detection of 0.5 fg/ml BoNT/A. This assay is currently being optimized for detection of BoNTs in complex animal tissues.

Previous research has suggested the following methods by which anti-toxin antibodies can neutralize toxin: inhibition of the catalytic domain and thus neutralization of the endoprotease activity of the Lc, inhibition of toxin binding to neuronal cells, or opsonization and clearance of the antibody-toxin complex from the bloodstream by cell processes (1, 25). Using two different in vitro methods, we demonstrated that neither MAb F1-2 nor F1-40 inhibited the endopeptidase activity, ruling out the possibility that neutralization occurred via inhibition of endopeptidase activity (Fig. 6A and B). In contrast, binding with either F1-2 or F1-40 prevented intracellular SNAP25 cleavage in either primary cortical neurons or cultured Neuro-2A neuroblastoma cells (Fig. 6C and data not shown). These data suggest that, via steric hindrance, MAbs could have interfered with BoNT/A receptor binding, subsequent neuron internalization, or interaction with intracellular SNAP25. F1-2, the MAb against the translocation domain of the toxin, was slightly more efficient in this inhibition than F1-40, the MAb against the Lc domain (Fig. 6C), presumably due to the lower affinity of F1-40 than F1-2 for BoNT/A (28). This was also observed in the lower neutralization potential of F1-40 than F1-2 (Fig. 1A and B). In addition, as suggestive of immune clearance, our MAb neutralization experiments, as well as a previous report, showed that BoNT/A was cleared from the serum within 10 min of MAb introduction, with the toxin subsequently found sequestered in the liver and spleen of the animal (21). Clearly, two likely mechanisms for MAb toxin neutralization are evident, (i) inhibiting toxin internalization by steric hindrance of toxin receptor binding and (ii) enhancing clearance by the immune system. Which mechanism is more predominant and efficacious in preventing establishment of botulism remains to be determined.

Future studies should pinpoint the various steps in the intoxication process, especially those involved in oral intoxication. Also important are studies of the onset of paralysis after

toxin internalization. Visible signs of paralysis were not evident until well after the $t_{1/2\alpha}$ (data not shown). One could potentially use muscle paralysis tests to pinpoint and map the time delay between absorption and onset of paralysis to estimate the time for toxin sequestration to paralysis. Additional areas that need to be explored include the rate of toxin absorption, saturation of toxin receptor binding sites, and the rate of recovery following toxin absorption.

This study aimed to carefully assess the contribution of MAbs with defined epitopes to toxin neutralization in oral and systemic animal intoxication models. Increased knowledge of BoNT antibody recognition or neutralization sites that could be amenable to toxin neutralization and the mechanism by which they contribute to toxin neutralization is the first stage in the design of better therapeutics and more efficacious treatment.

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